INFECTION AND IMMUNITY, Nov. 1986, p. 395–402
0019-9567/86/110395-08$02.00/0

Vol. 54, No. 2

DNA Sequence and Product Analysis of the *virF* Locus Responsible for Congo Red Binding and Cell Invasion in *Shigella flexneri* 2a

TAKASHI SAKAI, CHIHIRO SASAKAWA, SOUICHI MAKINO, AND MASANOSUKE YOSHIKAWA*

Department of Bacteriology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan

Received 8 April 1986/Accepted 14 July 1986

The DNA sequence of *virF*, a locus associated with virulence and the ability to bind Congo red in *Shigella flexneri* 2a that is located on a 140-megadalton (230-kilobase) plasmid, was determined and analyzed. It was rich in A and T. The direction of transcription of *virF* was determined by using a chloramphenicol resistance cartridge. An open reading frame readable in this direction was found. Three proteins, 30, 27, and 21 kilodaltons, all corresponding to those predicted from the above sequence, were produced in minicells containing the *virF* locus. The three proteins were expressed only weakly in minicells with the 230-kilobase plasmid.

Invasion by and multiplication of shigellae in colonic epithelial cells are the essential attributes in the early stage leading to bacillary dysentery (11). The genetic determinants for these attributes are located on a large 140-megadalton (MDa) (230-kilobase [kb]) plasmid (22, 23) as well as on the chromosome in *S. flexneri* (22). The ability of shigellae to bind Congo red has been implicated in their virulence (12, 25). This phenotype will be described as Pcr (pigmentation of Congo red). In *Shigella flexneri* 2a strain YSH6000, at least three genetic loci on the 230-kb plasmid have been shown to be essential for the Pcr^+ phenotype (26). They are located more than 70 kb apart from each other (26). Apparently in paradox to these observations, a region called *virF* of about 1 kb has been cloned in *Escherichia coli* K-12 by selecting for the Pcr^+ phenotype. Subsequently, *virF* has been found essential but not sufficient both for the Pcr^+ phenotype and for virulence in *S. flexneri* (19).

In the present study, the DNA sequence of *virF* and its product have been determined, and their characteristics are discussed.

MATERIALS AND METHODS

Isolation of plasmid DNA. Plasmid DNA was isolated as described previously (19).

Restriction endonuclease analysis and gel electrophoresis. The methods for restriction endonuclease analysis and gel electrophoresis have been described previously (19, 25). Sticky ends of pMYSH6509 made by cleaving with *Bam*HI were filled in with Klenow polymerase at 16°C for 2 h.

DNA ligation and transformation. The cleaved vector DNA and the DNA to be cloned were mixed and ligated with T4 ligase by incubating at 14°C for 12 h. The DNA molecules thus ligated were transformed into *E. coli* MC1061 (2) by the method of Morrison (16) or transferred to JM101 (13) or M2124 (28) by the method of Curtiss (4).

DNA sequence determination by the M13 dideoxy method. DNA fragments of the *virF* locus were cloned into M13mp8 (14) and transformed into JM101. Their DNA sequence was determined by the method of Sanger et al. (20, 21).

Measurement of MICs of chloramphenicol. Plasmid-carrying MC1061 was grown overnight at 30°C in LN broth (24) containing 100 μg of ampicillin per ml. One-twentieth milliliter of 100-fold dilutions of these cultures was inoculated into Penassay broth (Difco Laboratories, Detroit, Mich.) containing twofold serial dilutions of chloramphenicol. The results were read after 24 h of standing incubation at both 37 and 30°C and expressed as the MIC.

Construction of pMY6004. Since the expected molecular size of the *virF* product was similar to that of the beta-lactamase gene of pBR322, it was necessary to replace the ampicillin resistance gene of pBR322 with another drug resistance gene. First, a *Bgl*II linker was inserted into the *Hin*cII site of the ampicillin resistance gene, and the *Bgl*II-*Eco*RI fragment was replaced by the *Bam*HI-*Eco*RI fragment coding for trimethoprim resistance from R388 (29). The *Hin*dIII-*Sal*I fragment within the tetracycline resistance gene was removed, and the sticky ends were filled in by treatment with Klenow polymerase and then ligated with T4 ligase to yield vector pMY6004.

Construction of M2124, containing the 230-kb plasmid. The conintegrate (19) between pMYSH6000 and a replication-thermosensitive derivative of R388::Tn*5* was conjugally transferred into M2124. Since *S. flexneri* containing the cointegrate proved to be fully virulent and Pcr^+ (19), the virulence and Pcr^+ determinants of the plasmid had not been inactivated during cointegrate formation. The transconjugant was then cured of a replication-thermosensitive derivative of R388::Tn*5* by growth in drug-free LN broth at 42°C. A derivative of M2124 which was kanamycin sensitive and trimethoprim sensitive, subsequently confirmed by molecular analysis and its Pcr^+ phenotype to be pMYSH6000::IS*50*, was selected.

Analysis of plasmid-coded proteins. Minicells from 250-ml stationary-phase cultures of strain M2124 containing pMY6004 or pMYSH6513 were grown at 37°C in Mueller Hinton broth (Difco Laboratories, Detroit, Mich.) containing 5 μg of trimethoprim. M2124 containing pMYSH 6000::IS*50* was grown at 37°C in LN broth. Minicells were separated by the method of Andrés et al. (1), preincubated at 30 or 37°C for 1 h in methionine assay-minimal glucose salts

* Corresponding author.

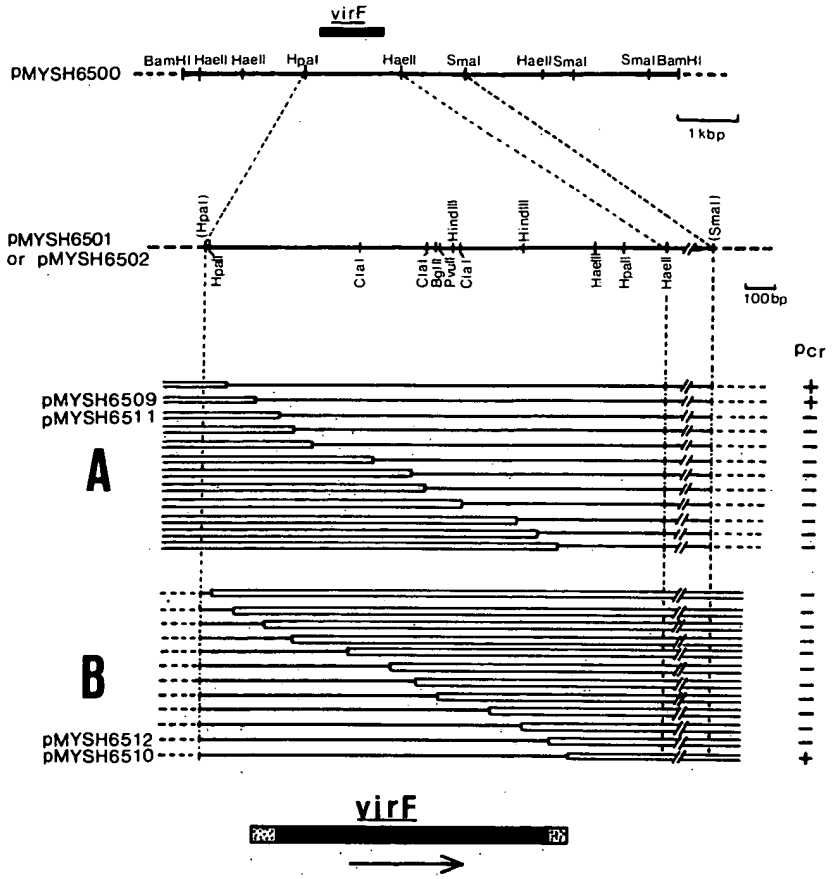


FIG. 1. Deletion map of the *virF* locus. (A) Deletions derived from pMYSH6501; (B) deletions derived from pMYSH6502 (19). ——, 230-kb plasmid fragment; -----, pBR322 vector; ▭, deleted regions; ▩, regions containing *virF* termini. The arrow shows the reading direction of the *virF* locus. There is a linker-derived *Bam*HI site at the end of each deletion. Previous restriction sites for the enzymes shown which now cannot be cut again because of manipulation of these sites are shown in parentheses.

medium (9), labeled with [^{35}S]methionine at 30 or 37°C for 4 h, collected by centrifugation, and incubated in LN broth containing 1.5% caseine hydrolysate at 30 or 37°C for 10 min. The collected minicells were lysed by the method of Andrés et al. (1) and analyzed by electrophoresis in a 14% polyacrylamide gel containing sodium dodecyl sulfate.

DNA-DNA hybridization. The method used for DNA-DNA hybridization has been described previously (19). In brief, filters were hybridized at 42°C with a hybridization mixture containing 0.6 M NaCl, 0.2 M Tris hydrochloride (pH 7.9), 0.02 M EDTA, 0.5% sodium dodecyl sulfate, and 60% formamide.

RESULTS

DNA sequence analysis of *virF*. The minimum *virF* region has been determined by making Bal31 deletions from the *Bam*HI site on the pBR322 part of pMYSH6501 and pMYSH6502 (19) (Fig. 1) and by determining their Pcr phenotype. The deletion ends of all these mutants had been linked to a *Bam*HI linker in the previous study (19). Therefore, various parts of the *virF* locus could be cleaved by double digestion with *Eco*RI for the site on the pBR322 vector and *Bam*HI for the site on the linker at the deletion end. These fragments were recloned to an M13mp8 vector. The deletion ends of the mutants chosen were separated by 50 to 250 bp and derived from two plasmids with the *virF* locus in opposite orientations, as shown in Fig. 1. It was therefore rather easy to read the sequences bidirectionally and duplicately by determining 200- to 300-bp sequences from each M13 clone. The sequence listed in Fig. 2 is shown in the 5′ to 3′ direction to correspond to the direction of the arrow in Fig. 1.

Direction of transcription of *virF*. A chloramphenicol-resistance cartridge (3) containing the structural gene coding for chloramphenicol acetyltransferase but without the promoter was inserted into the *Bgl*II site of pMYSH6509 (Fig. 1). The cartridge was inserted from the left to the right in pMYSH6514 with respect to the direction shown in Fig. 1 and in the opposite direction in pMYSH6515. When transformed into *E. coli* K-12 strain MC1061, the MIC of chloramphenicol for the strain carrying pMYSH6514 was 100 μg/ml at 37°C and 50 μg/ml at 30°C, whereas the MIC for the strain carrying pMYSH6515 was 6.25 μg/ml at both 37 and 30°C. This was in spite of the possible readthrough in the latter strain from the promoter for tetracycline resistance on the vector located upstream from the cartridge.

Analysis of protein products of *virF*. A cloned large fragment containing *virF* had been shown to produce a ca. 20-kilodalton (kDa) protein (19). Its molecular size was determined to be 21 kDa by more precise gel analysis (described below). Furthermore, the cloned *virF* region had

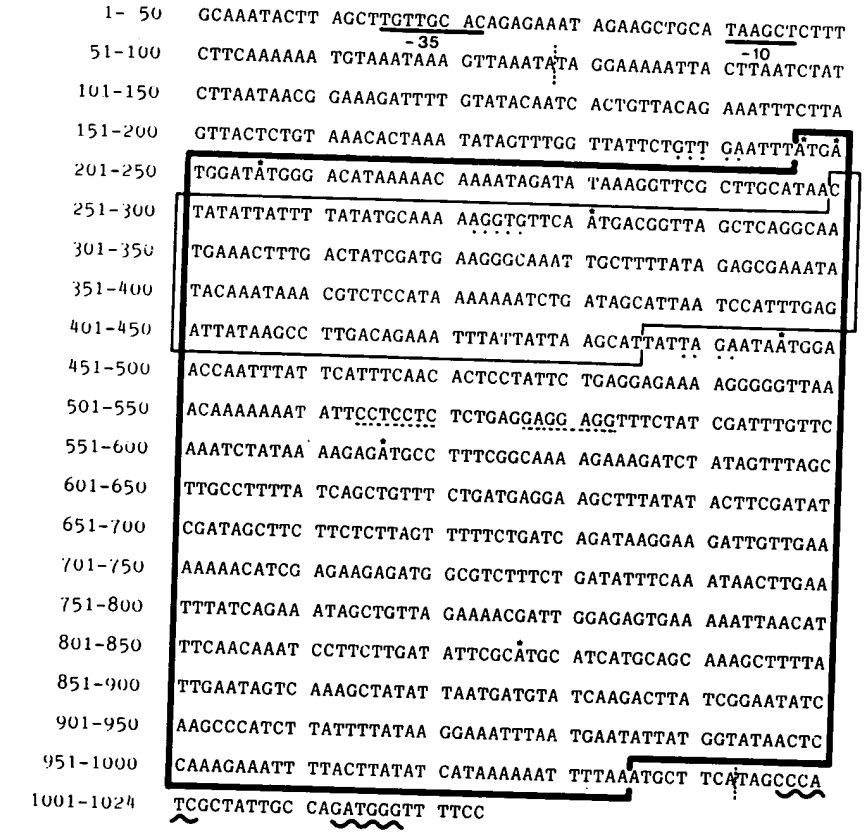


FIG. 2. DNA sequence of the *virF* locus. The DNA sequence from the deletion end of pMYSH6509 to that of pMYSH6510 in the direction shown in Fig. 1 is shown. Boxed areas, Open reading frames; heavy line, codes for 262 amino acids; thin line, codes for 61 amino acids. The promoters in the −35 region and −10 sequence are underlined with a straight line; inverted repeats followed by four continuous T's presumably terminators, are underlined with a wavy line. Vertical dashed lines indicate deletion ends of Pcr⁻ derivatives pMYSH6511 and pMYSH6512; Tentative attenuator sequences are underlined with a dashed line. ★, possible methionine codons of the large open reading frame. Probable ribosome binding sites (Shine-Dalgarno sequences) are shown with dots beneath.

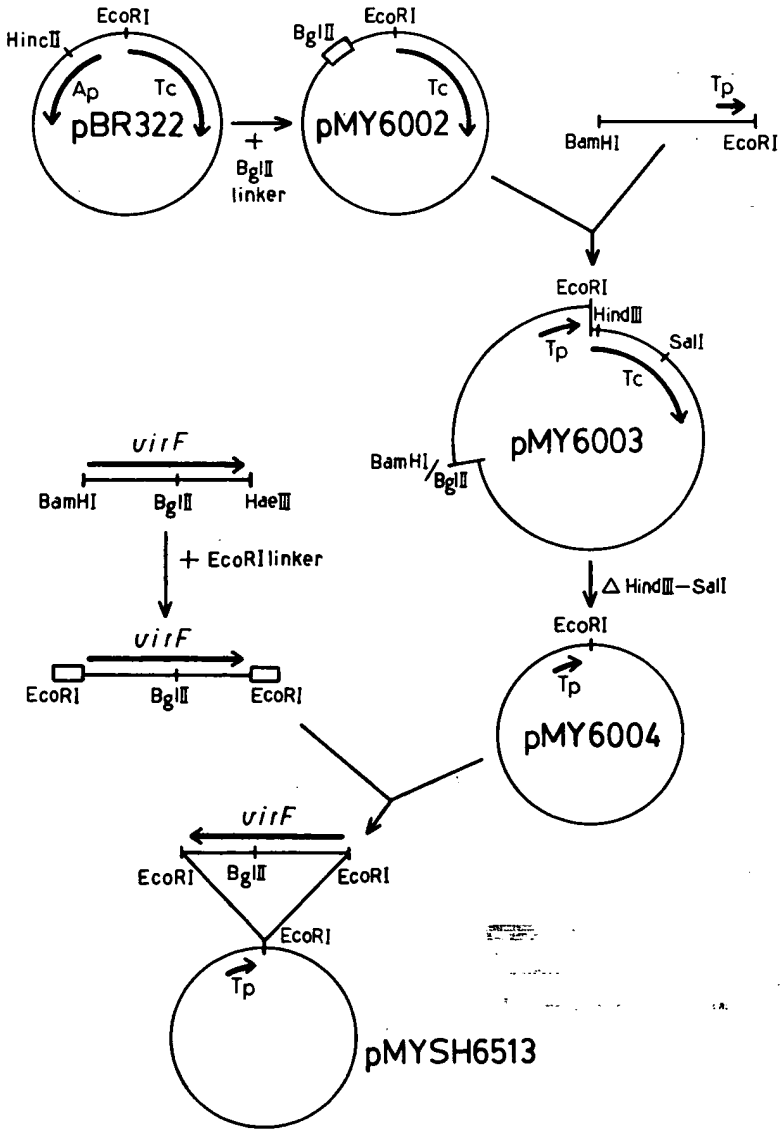


FIG. 3. Construction of pMYSH6513. Ap, Coding region for ampicillin resistance from pBR322; Tc, coding region for tetracycline resistance from pBR322; Tp, coding region for trimethoprim resistance from R388. The *virF* fragment was derived from pMYSH6509. Open boxes, Sites derived from linkers.

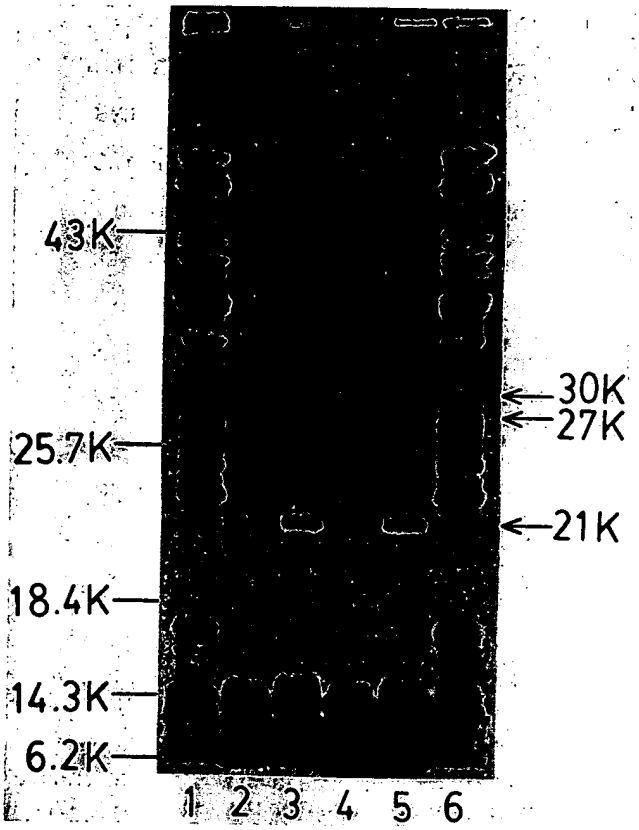


FIG. 4. Protein analysis by the minicell method. Lanes 1 and 6, pMYSH6000::IS*50*; lanes 2 and 4, pMY6004; lanes 3 and 5, pMYSH6513. Lanes 1, 2, and 3, growth at 30°C; lanes 4, 5, and 6, growth at 37°C. Numbers at the sides indicate molecular sizes (in kilodaltons).

been complemented in *trans* both for Pcr$^+$ and for virulence when it coexisted in an *S. flexneri* 2a YSH6000 derivative with a small deletion in the *Sal*I fragment F of the 230-kb plasmid (19). To analyze the product more accurately, a fragment with the *Bam*HI site of the linker at one end and the *Hae*III site at the other was cleaved from pMYSH6509 (Fig. 1 and 3), the *Bam*HI site was filled in, and then both ends were linked to an *Eco*RI linker. This small *virF* fragment with *Eco*RI sites at both termini was linked to the *Eco*RI site of a pBR322-derived trimethoprim resistance vector, pMY6004, to obtain pMYSH6513 (Fig. 3). A minicell analysis with pMYSH6513 and its vector, pMY6004, as a control revealed the production of proteins of about 30 and 27-kDa at both 30 and 37°C in addition to one of 21 kDa identified previously (19) (Fig. 4). These three proteins were expressed only weakly on the 230-kb plasmid.

Interpretation of DNA sequence of *virF*. Since the 5′ end of the DNA sequence shown in Fig. 2 is the deletion end of pMYSH6509, and the 3′ end corresponds to the deletion end of pMYSH6510, this sequence is considered the minimum required for the Pcr$^+$ phenotype in *E. coli* K-12. One of the striking characteristics of the sequence is the richness in A and T. The GC content is about 30%. The open reading frame found within this sequence codes for 262 amino acid residues (boxed with thick lines in Fig. 2), and another codes for 61 amino acid residues in the opposite direction (boxed with thin lines in Fig. 2). Since the deletion mutations

TABLE 1. Codon usage in *virF* and in *E. coli*

Amino acid	Codon	Frequency per 1,000 codons		
		virF	*E. coli* ribosomal[a]	*E. coli* mix[b]
Arg	AGA	23	1	2
	AGG	15	0	0
	CGA	8	0	4
	CGU	4	42	19
	CGC	8	22	17
	CGG	0	1	2
Leu	UUA	49	4	10
	UUG	23	3	9
	CUA	0	0	4
	CUU	31	4	8
	CUC	8	3	8
	CUG	0	61	70
Ser	AGU	15	4	5
	AGC	23	6	13
	UCA	31	1	7
	UCU	42	22	19
	UCC	8	19	13
	UCG	8	2	10
Pro	CCA	15	5	8
	CCU	4	4	4
	CCC	0	1	7
	CCG	0	29	19
Ala	GCA	11	38	22
	GCU	23	68	15
	GCC	0	12	28
	GCG	0	25	42
Gly	GGA	8	1	4
	GGU	4	40	32
	GGC	8	32	37
	GGG	8	0	5
Thr	ACA	4	3	2
	ACU	15	28	10
	ACC	0	19	23
	ACG	4	2	13
Val	GUA	4	38	13
	GUU	23	43	23
	GUC	4	7	13
	GUG	0	15	28
Ile	AUA	65	1	1
	AUU	46	15	33
	AUC	23	34	35
Gln	CAA	23	7	15
	CAG	4	24	31
His	CAU	19	4	7
	CAC	4	8	7
Glu	GAA	31	53	32
	GAG	42	14	20
Asp	GAU	38	13	25
	GAC	4	28	18
Tyr	UAU	42	4	11
	UAC	4	12	14
Phe	UUU	34	8	30
	UUC	11	15	22
Lys	AAA	69	70	40
	AAG	23	25	9
Asn	AAU	34	4	13
	AAC	23	29	25
Cys	UGU	4	1	6
	UGC	4	5	6
Met	AUG	27	22	25
Trp	UGG	4	4	6

[a] Data from Post and Nomura (17).
[b] Data from Greene et al. (7).

MET MET ASP MET GLY HIS LYS ASN LYS ILE ASP ILE LYS VAL ARG LEU HIS ASN TYR ILE
ILE LEU TYR ALA LYS ARG CYS SER MET THR VAL SER SER GLY ASN GLU THR LEU THR ILE
ASP GLU GLY GLN ILE ALA PHE ILE GLU ARG ASN ILE GLN ILE ASN VAL SER ILE LYS LYS
SER ASP SER ILE ASN PRO PHE GLU ILE ILE SER LEU ASP ARG ASN LEU LEU LEU SER ILE
ILE ARG ILE MET GLU PRO ILE TYR SER PHE GLN HIS SER TYR SER GLU GLU LYS ARG GLY
LEU ASN LYS LYS ILE PHE LEU LEU SER GLU GLU GLU VAL SER ILE ASP LEU PHE LYS SER
ILE LYS GLU MET PRO PHE GLY LYS ARG LYS ILE TYR SER LEU ALA CYS LEU LEU SER ALA
VAL SER ASP GLU GLU ALA LEU TYR THR SER ILE SER ILE ALA SER SER LEU SER PHE SER
ASP GLN ILE ARG LYS ILE VAL GLU LYS ASN ILE GLU LYS ARG TRP ARG LEU SER ASP ILE
SER ASN ASN LEU ASN LEU SER GLU ILE ALA VAL ARG LYS ARG LEU GLU SER GLU LYS LEU
THR PHE GLN GLN ILE LEU LEU ASP ILE ARG MET HIS HIS ALA ALA LYS LEU LEU LEU ASN
SER GLN SER TYR ILE ASN ASP VAL SER ARG LEU ILE GLY ILE SER SER PRO SER TYR PHE
ILE ARG LYS PHE ASN GLU TYR TYR GLY ILE THR PRO LYS LYS PHE TYR LEU TYR HIS LYS
LYS PHE

FIG. 5. Amino acid sequence predicted from the large open reading frame. Within the large open reading frame, seven methionine codons are found (boxed).

extending inside the vertical broken lines at 78 base pairs (bp) (corresponding to the deletion end of pMYSH6511) and 993 bp (corresponding to the deletion end of pMYSH6512) gave rise to Pcr$^-$, the sequences between 1 and 78 and that between 993 and 1024 bp are essential for Pcr$^+$. Within the former sequence, the -35 region and -10 sequence (Pribnow box) can be seen (solid underline) (18), but no clear ribosome binding site (27) was found. Within the latter sequence there was a typical terminator (18), consisting of GC-rich inverted repeats (waved underline) with four continuous T's in the 3′ direction. No promoterlike sequence was found for the opposite reading frame for 61 amino acid residues in the latter sequence. This is consistent with the evidence obtained by the chloramphenicol cartridge. The amino acid sequence coded by the larger open reading frame is shown in Fig. 5. Since the nucleotide sequence is rich in A and T, the frequencies of codon usage are also strikingly characteristic compared with those of *E. coli* (7, 17), (Table 1).

DISCUSSION

The data presented in this study are compatible with the interpretation that the DNA sequence shown in Fig. 2 (boxed with thick solid lines) is transcribed from the left to the right. A -35 region and a -10 sequence (Pribnow box) were found. However, no clear ribosome binding site was found. It should be pointed out in this connection that either one sequence or the other preceding the first, fourth, and fifth ATG codons may be the ribosome binding site. Corresponding to these initiation codons, proteins of about 30, 27, and 21 kDa may be produced. Their molecular sizes correspond exactly to those produced by the minicells.

During a search for the ribosome binding site, we found a characteristic sequence (shown by broken underlines in Fig. 2). It is an attenuator sequence (10) consisting of GC-rich inverted repeats with three continuous T's in the 3′ direction. The downstream strand of the repeats is a typical ribosome binding site (27). These structures may regulate the production of these proteins. If this ribosome binding site is really functional, a 16-kDa protein would be produced. No such protein was found in the minicell analysis (Fig. 4). This is presumably because the ribosome binding site is blocked due to the stem-loop structure.

For the possible proteins translated from the first, second, and third ATG codons, a signal peptide-like amino acid sequence (15) was seen at their N terminus (amino acids 1 to 24). If the signal peptide is cleaved during passage through the inner membrane, the resulting protein may become about 27 kDa.

Hale et al. (8) reported that proteins of 20, 25, 38, 43, 53, 62, and 78 kDa were associated with virulence in *Shigella* spp. and enteroinvasive *Escherichia coli* and that they were in a repressed state in organisms grown at 30°C. At this moment it cannot be decided which of the 30-, 27-, and 21-kDa proteins we found is really responsible for the Pcr$^+$ phenotype. We also cannot decide the correlation between the proteins found by Hale et al. (8) and by us.

The secondary structure of the protein coded by this large open reading frame was analyzed by the method of Garnier (5) with a computer program. Figure 6 shows the location of the predicted alpha-helix, beta-sheet, and beta-turn conformations. This protein is highly characteristic in its rich beta-sheets. Its helical content was estimated to be 36%. It has been shown that Congo red staining of the amyloid-fibril protein deposited in amyloidosis is dependent on the beta-pleated sheet configuration (6). If the *virF* product is really rich in beta-sheet, a similar direct interaction between Congo red and the *virF* protein may be responsible for the Pcr phenotype.

Finally, Southern hybridization analysis with a probe made by cleaving pMYSH6509 with *Bam*HI and *Hae*III has

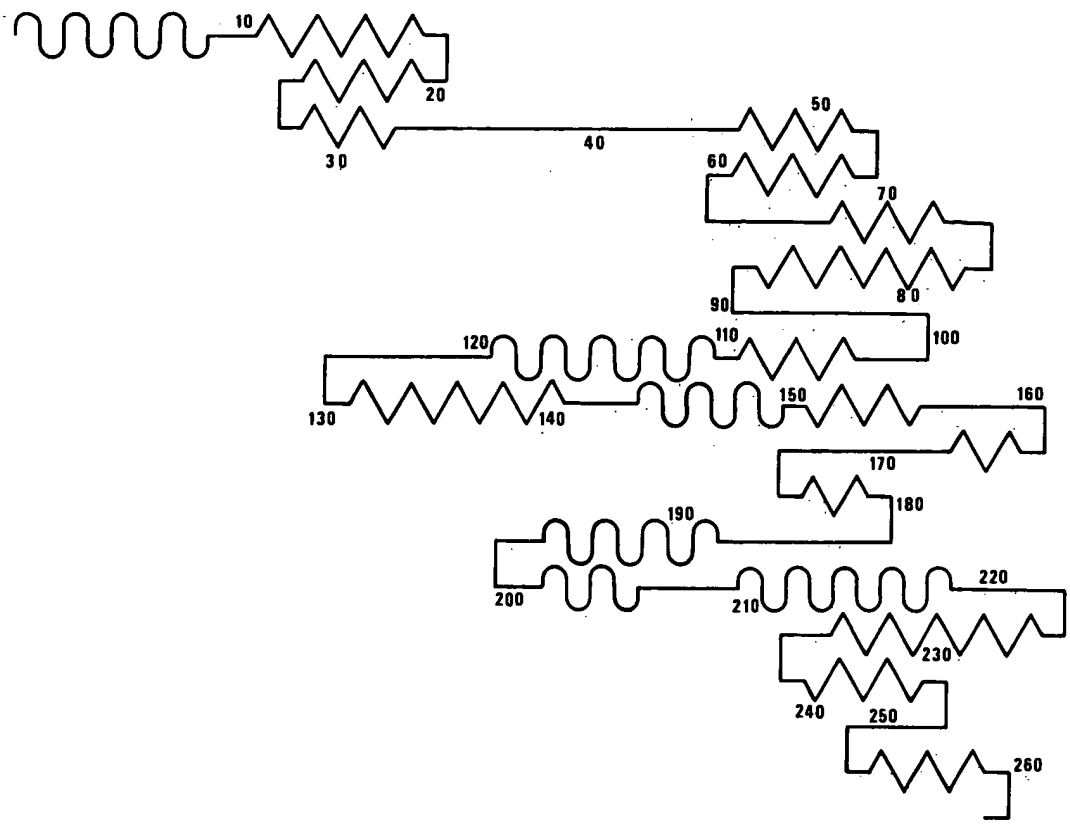


FIG. 6. Prediction of the secondary structure of the large open reading frame. Alpha-helix, beta-sheet, and beta-turn are shown by wavy lines, jagged lines, and end brackets, respectively. Numbers show amino acid residues from the N terminus.

revealed that the large virulence plasmids of *S. flexneri* 2a, 2b, 3a, 3b, 3c, 4, 5, and 6, *S. dysenteriae*, *S. boydii*, *S. sonnei*, and enteroinvasive *E. coli* have a similar sequence, although weak nonspecific hybridization was encountered, presumably due to the AT richness (data not shown). These observations indicate that the *virF* protein is required for the invasion process of all these pathogens.

ACKNOWLEDGMENT

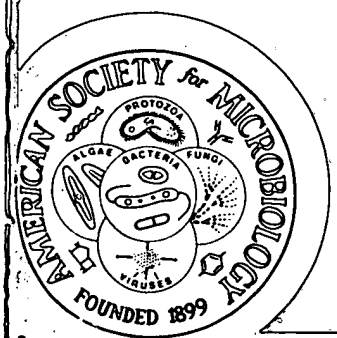
This study was supported by grants B59480159 and A61440035, provided by the Ministry of Education, Science, and Culture of the Japanese government.

LITERATURE CITED

1. **Andrés, I., P. M. Slocombe, F. Cabello, J. K. Timmis, R. Lurz, H. J. Burkardt, and K. N. Timmis.** 1979. Plasmid replication functions. Mol. Gen. Genet. **168**:1–25.
2. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
3. **Close, T. J., and R. L. Rodriguez.** 1982. Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptional mapping of extrachromosomal elements. Gene **20**:305–316.
4. **Curtiss, R., III.** 1981. Transformation in *Escherichia coli*, p. 247–249. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
5. **Garnier, J.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
6. **Glenner, G. G.** 1980. Amyloid deposits and amyloidosis. N. Engl. J. Med. **302**:1283–1292.
7. **Greene, P. J., M. Gupta, H. W. Boyer, W. E. Brown, and J. M. Rosenberg.** 1981. Sequence analysis of the DNA encoding the *Eco*RI endonuclease and methylase. J. Biol. Chem. **256**: 2143–2153.
8. **Hale, T. L., E. V. Oaks, and S. B. Formal.** 1985. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. Infect. Immun. **50**:620–629.
9. **Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Austin, and S. B. Formal.** 1983. Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect. Immun. **40**: 340–350.
10. **Kolter, R., and C. Yanofsky.** 1982. Attenuation in amino acid biosynthetic operons. Annu. Rev. Microbiol. **16**:113–134.
11. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
12. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. **43**:397–401.
13. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. Part C **101**:20–78.
14. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digested restriction fragments. Gene **19**:269–276.
15. **Michaelis, S., and J. Beckwith.** 1982. Mechanism of incorpora-

tion of cell envelope proteins in *Escherichia coli*. Annu. Rev. Microbiol. **36**:435–465.
16. **Morrison, D. A.** 1977. Transformation in *Escherichia coli*: cryogenic preservation of competent cells. J. Bacteriol. **132**:349–351.
17. **Post, L. E., and M. Nomura.** 1980. DNA sequences from the *str* operon of *Escherichia coli*. J. Biol. Chem. **255**:4660–4666.
18. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
19. **Sakai, T., C. Sasakawa, S. Makino, K. Kamata, and M. Yoshikawa.** 1986. Molecular cloning of a genetic determinant for Congo red binding ability, which is essential for the virulence of *Shigella flexneri*. Infect. Immun. **51**:476–482.
20. **Sanger, F.** 1981. Determination of nucleotide sequences in DNA. Science **214**:1205–1210.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
22. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
23. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
24. **Sasakawa, C., and D. E. Berg.** 1982. IS*50*-mediated inverse transposition: discrimination between the two ends of an IS element. J. Mol. Biol. **159**:257–271.
25. **Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa.** 1986. Molecular alteration of the 140-megadalton plasmid associated with the loss of virulence and Congo red binding activity in *Shigella flexneri*. Infect. Immun. **51**:470–475.
26. **Sasakawa, C., S. Makino, K. Kamata, and M. Yoshikawa.** 1986. Isolation, characterization, and mapping of Tn*5* insertions into the 140-megadalton invasion plasmid defective in the mouse Sereny test in *Shigella flexneri* 2a. **54**:32–36.
27. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
28. **Thompson, R., and M. Achtman.** 1978. The control region of the F sex factor DNA transfer cistrons: restriction mapping and DNA cloning. Mol. Gen. Genet. **165**:295–304.
29. **Zolg, J. W., U. J. Hanggi, and H. G. Zachau.** 1978. Isolation of a small DNA fragment carrying the gene for a dihydrofolate reductase from a trimethoprim resistance factor. Mol. Gen. Genet. **164**:15–29.

Infection and Immunity



Published monthly by the
American Society for Microbiology

CODEN: INFIBR 54 (2) 273–602 (1986) ISSN: 0019-9569